

PB83-116962

Determination of Benzidines by Gas
Chromatographic Separation of
Derivatives with Electron
Capture Detection

(U.S.) Environmental Monitoring and Support Lab.
Cincinnati, OH

1982



U.S. Department of Commerce
National Technical Information Service

NTIS

TECHNICAL REPORT DATA		
(Please read instructions on the reverse before completing)		
1. REPORT NO. EPA-600/J-82-011	2. JOURNAL ARTICLE	3. PB83-116962
4. TITLE AND SUBTITLE Determination of Benzidines by Gas Chromatographic Separation of Derivatives with Electron Capture Detection	5. REPORT DATE 1982	6. PERFORMING ORGANIZATION CODE
	8. PERFORMING ORGANIZATION REPORT NO.	
7. AUTHOR(S) F.K. Kawahara, J.R. Dunn, F.A. Fuitem, P.J. McCullough	10. PROGRAM ELEMENT NO. CBEB1C	11. CONTRACT/GRANT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Protection Agency Environmental Monitoring and Support Laboratory 26 W St. Clair Cincinnati, OH 45268	13. TYPE OF REPORT AND PERIOD COVERED journal reprint	
	14. SPONSORING AGENCY CODE EPA 600/06	
12. SPONSORING AGENCY NAME AND ADDRESS same as above		
15. SUPPLEMENTARY NOTES REF: Analytica Chimica Acta, 138:207-220, 1982		
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17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) unclassified	21. NO. OF PAGES 15
	20. SECURITY CLASS (This page) unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

Analytica Chimica Acta, 138 (1982) 207-220

Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

DETERMINATION OF BENZIDINES BY GAS CHROMATOGRAPHIC SEPARATION OF DERIVATIVES WITH ELECTRON CAPTURE DETECTION^a

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(Received 12th October 1981)

SUMMARY

Factors considered in the development of a method for the determination of toxic, water-soluble benzidines, involved providing a good technique for their adequate recovery, converting quantitatively the isolated amines to relatively stable derivatives suitable for measurement and storage, coupled with relative ease of preparation of the derivatives, and resolving the prepared compounds during their measurement. Benzidine and dichlorobenzidine are quantitatively extracted from wastewater and measured quantitatively through the preparation of their respective pentafluoropropionamides (PFP) by using PFP-imidazole. Overall recovery efficiency for benzidines from wastewater ranges from 91 to 103%. These derivatives are relatively stable compounds and have detection limits of 0.2 pg or less, when electron capture detection is used with gas chromatography. Derivatives of *o*-tolidine and dianisidine are also described.

The discharge of hazardous industrial wastes into natural waters has become a serious environmental concern. These substances pose a toxic threat to the ecosystem and to public health. The U.S. Environmental Protection Agency has, therefore, established a list of hazardous chemicals, or "priority pollutants". When found in effluents, they demand monitoring for control. Thus arises the need for accurate and sensitive methods for the routine determination of these compounds [1, 2].

Two priority pollutants, benzidine and 3,3'-dichlorobenzidine, have been used in azo dye synthesis. Epidemiological evidence leaves little doubt that exposure to benzidine can lead to bladder cancer in humans. Furthermore, Wendel et al. [3] reported 62% of industrial workers exposed to benzidine over a 13-year period had developed urinary bladder cancer. Though there is currently no evidence to indicate that the benzidine congener, 3,3'-dichlorobenzidine, causes bladder cancer, it may nevertheless be implicated as a carcinogen because it is also a chloroaromatic compound containing a vinyl chloride grouping in its aromatic structure. The carcinogenicity of vinyl chloride in rats was shown in 1970 [4, 5]. Male mice exposed to technical

^aMention of trade names or commercial products does not constitute endorsement or recommendation for use.

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grade 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl) ethane (DDT), containing two vinyl groupings in its aromatic moiety, suffer from a large incidence of hepatomas at even 2 ppm [6]. In addition to their carcinogenic potential, the benzidines are known to have toxic effects causing dermatitis and cystitis in humans.

Various methods for the determination of benzidines have been reported, and the chloramine-T spectrophotometric method is the oldest. Having detection limits in the ppb range, good precision, and moderate accuracy, the chloramine-T method [7] is still limited, because it cannot differentiate between benzidine and its congeners. Though thin-layer chromatography is a simple, frequently used, quantitative method for separation and identification of benzidine and its congeners, [8] it is relatively insensitive. Spectrofluorimetry has also been explored as a method for benzidine determination [9]. Yet, the determination of mixed benzidine congeners is not feasible because of spectral overlap and varying absorption intensities.

Recently, Riggan and Howard, and others, developed a high performance liquid chromatographic (h.p.l.c.) method using solvent extraction for the determination of benzidine, 3,3'-dichlorobenzidine and 1,2-diphenylhydrazine in aqueous medium [10-12]. To increase sensitivity and selectivity, these authors attempted perfluoroacylation derivatization of the above compounds and found that they could prepare only the 3,3'-dichlorobenzidine derivative with pentafluorobenzoyl chloride.

Heptafluorobutyrylimidazole (HFBI) and trifluoroacetylimidazole (TFAI) originally synthesized by Staab and Walther [13] had been used to prepare the HFB and TFA derivatives of the methyl esters of gibberellins, endogenous plant growth hormones. These derivatives made possible on a routine basis the determinations by gas chromatography (g.c.) of ultratrace amounts of hormones [14]. Bradway and Shafik [15] evaluated the derivatization techniques currently available for the g.c. determination of substituted anilines. Their use of HFBI produced a broad tailing solvent front. This problem was largely corrected by washing with dilute hydrochloric acid solution. Horning et al. [16] described a g.c. method for the characterization and separation of aromatic and aliphatic amines using the pentafluoropropionyl derivatives. A g.c. method for the simultaneous determination of *o*-, *m*-, and *p*-aminophenol and aniline in water was described by Coutts et al. [17]; it involved a two-step acetate derivatization technique of these amines. Currently, however, there is no suitable g.c. method for the ultratrace determination of benzidines taken from water media.

The purpose of this study was to investigate various types of perfluoroacylation of benzidine and 3,3'-dichlorobenzidine, with subsequent detection by electron capture gas chromatography. These methods were chosen in order to improve the sensitivity and selectivity of the determinations, and to provide a means for the determination of other primary and secondary amines which may be hazardous.

EXPERIMENTAL

Reagents

Benzidines of 97% purity were obtained (K&K-ICN Laboratories, Plainview, New York). Sealed glass ampules (1 g) of the perfluoroacylating reagents, *N*-methyl-bis-(trifluoroacetamide), *N*-trifluoroacetylimidazole (TFAI), and *N*-pentafluoropropionylimidazole (PFPI) were obtained in 20-g bottles (Pierce Chemical Co., Rockford, IL); any formation of a pink to red color appears to indicate that the TFAI reagent may be of unusable quality. Other reagents used were trifluorothioacetic acid (PCR, Gainesville, FL), dieldrin (Pesticides and Toxic Substance Effects Laboratory, National Environmental Research Center, USEPA, Research Triangle Park, NC), and pesticide-grade organic solvents, methylene chloride, chloroform, carbon tetrachloride, toluene, dimethylformamide, and ethyl acetate (Burdick and Jackson, Muskegon, MI).

To remove residual water effectively from the solvents, the initial 15% of distillate was discarded whenever carbon tetrachloride, chloroform, methylene chloride, or toluene was distilled in glass. Water is too soluble in ethyl acetate for this method to dry the solvent efficiently. In this study, water was removed from the solvents or reaction mixtures with excess of pentafluoropropionylimidazole reagent, which reacts rapidly with water to form the free acid and free base which then react to form an insoluble white salt.

Apparatus

An all-glass vacuum distillation system with ground-glass fittings can be used to remove solvents after the derivatization reaction. A rotary vacuum apparatus (Rotavapor R-110; Brinkmann Instruments, Westbury, New York) is more convenient for the rapid removal of solvents and the PFPI reagent.

Perkin-Elmer gas chromatographs, Models 900 and 3920B, both equipped with ^{63}Ni electron capture detectors, were used. A glass column, 6 ft $\times$ 2 mm i.d., was initially employed utilizing 3% OV-17 on 100/120 mesh, Chromosorb WAW-DMCS, at 200°C. The injector temperature was set at 250°C, and the electron capture detector temperature was 300–350°C for the wastewater samples. Argon/methane (95%/5%) was used as the carrier gas at a flow rate of 20 ml min⁻¹. The attenuation of the model 900 was set at 32 $\times$ 10 and 64 $\times$ 10. For the 3920B model, the attenuation was set at $\times$ 256, $\times$ 512, and $\times$ 1024. The recorder chart speed was 1.0 cm min⁻¹ for the 3920B and 0.5 cm min⁻¹ for the 900 model. The range was 1 mV for both instruments. The 3920B was operated at 2 nA standing current with a pulse width of 250 ns. The 900 model was operated in a pulse mode with a pulse width of 25 μ s. Dieldrin was used as an external standard in the initial part of the study.

For the complete separation of benzidine PFP from dichlorobenzidine PFP, a 6 ft $\times$ 2 mm i.d. glass column with 45% of 3% OV-17 on Chromosorb

WAW-DMCS 100/120 mesh, 45% of 3% OV-210 on Chromosorb WAW-DMCS, 80/100 mesh, and 10% of 3% OV-225 on Chromosorb WHP, 100/120 mesh, was used. The sodium sulfate drying column, 30 cm $\times$ 1 cm, was fitted with a ground glass frit.

The gas chromatographs were interfaced to a Varian Aerograph 220/240 chromatographic data system. Mass spectral data were recorded on either a Finnigan 4000 gas chromatograph/mass spectrometer (g.c./m.s.) or a Hewlett-Packard Model 5993B g.c./m.s.

Procedure

Preliminary reactions were run on pure reagent materials without utilizing the extraction step, in order to determine the amount of reagent and the conditions necessary to convert compounds quantitatively to their derivative forms. Derivatives were then prepared from the extracts of wastewater and tap water spiked with the benzidine compounds in order to determine their recovery from a water matrix.

Reference standards were prepared from purified crystalline benzidine and were used to determine the degree of linearity, the useful operating range, and the percent recovery from various aqueous media.

Benzidine and dichlorobenzidine, as the salt-free, recrystallized amines, were dissolved separately in carbon tetrachloride or in chloroform to the 10 $\mu\text{g ml}^{-1}$ or 2 $\mu\text{g ml}^{-1}$ concentration levels. Ethyl acetate was added in minimal amounts in order to effect complete solution. *N*-Trifluoroacetyl-imidazole was prepared in carbon tetrachloride at a concentration of 300 mg ml^{-1} , while *N*-pentafluoropropionylimidazole (PFPI) was prepared in toluene at concentration levels of 250 to 500 mg ml^{-1} . To prepare PFPI at 500 mg ml^{-1} , 20 g of the commercial product was dissolved in 20 ml of toluene contained in a 50-ml brown bottle fitted with a bakelite cap having an 11-mm opening. The opening was sealed with a teflon rubber laminated disc, facilitating the removal of sample with a hypodermic syringe.

Micropreparation of derivatives

The benzidine derivatives were prepared in the following manner: 1 ml of benzidine solution (10 or 2 μg), 0.50 ml of PFP-imidazole solution (250 mg of reagent), 1.0 ml of dry methylene chloride or chloroform, and four drops of pyridine (150 mg) were added to a 50-ml round-bottom flask. The joint of the flask was lubricated lightly with Apiezon N grease applied to the standard tapered glass stopper which was secured in place with rubber bands. The mixture was heated at 60°C in a hot water bath for one hour. After the reaction mixture had cooled to room temperature, all solvents were removed by vacuum distillation at 40°C and 1 mm pressure. The remaining residue was macerated in a mixture of 9.0 ml of toluene and 1.0 ml of ethyl acetate.

After standing for 5 min, the 10 ml of solution containing the residue derivative was decanted into a separatory funnel and 7 ml of the toluene and ethyl acetate mixture (9:1) was added to the residue flask. Five drops (180

mg) of sodium thiosulfate solution containing 25 mg per 100 ml were added. After vigorous shaking, the contents were transferred to a separatory funnel. The mixture in the separatory funnel was not shaken, but rather was rolled very gently 10 times. Then the water was separated and decanted and the organic phase was dried by passing through a 2 cm $\times$ 0.8 cm anhydrous sodium sulfate column. The column was rinsed with 1.0 ml of toluene. After drying, the total volume of the percolate was adjusted to 20 ml. Results obtained by electron capture gas chromatography are given in Table 1 for pure reagent conversions.

Bulk preparation of derivatives

Dichlorobenzidine derivative was prepared in the following manner: 11 ml of chloroform, 51 mg of salt-free, recrystallized dichlorobenzidine (m.p. 134–136°C; Heilbron, m.p. 133°C) and 1.8 g of PFPI in 6 ml of toluene were added to a 50-ml round-bottom flask. After the mixture had been heated for 3 h at 90°C in a hot water bath, it was subjected to rotary vacuum evaporation. The resulting residue was partially dissolved with 30 ml of toluene and ethyl acetate mixture at a 9:1 ratio, and this mixture was transferred to a separatory funnel. A portion (25 ml) of the same toluene and ethyl acetate mixture was added to the residue in the flask followed by 4 ml of chlorine-free distilled water, and the mixture was gently shaken 10 times. The contents were then transferred to the separatory funnel containing the first 30-ml extract. The total mixture was then gently shaken 10 times in the separatory funnel and allowed to settle. The organic layer was passed through a 5.0 cm $\times$ 1.0 cm column of anhydrous sodium sulfate via dropwise addition from the separatory funnel, and the drying column was then washed with 5 ml of toluene. The filtrate, which had been collected in a 100-ml flask, was concentrated by rotary vacuum evaporation, producing a white residue of dichlorobenzidine PFP. When recrystallized once from a mixture of toluene, ethyl acetate and hexane (9:1:1), the crystals melted at 187–188°C and 101 mg of derivative was recovered. Standards were then prepared from these purified crystals at levels of 170–680 $\text{pg } \mu\text{l}^{-1}$ as the dichlorobenzidine. Mass spectra were obtained on the original and the derivatized dichlorobenzidine.

The benzidine derivative was prepared in the following manner: 119 mg of benzidine (m.p. 124–126°C) was added to a 50-ml flask with 25 ml of chloroform, 3.3 g of PFPI in 6.6 ml of toluene, and 1 ml of pyridine. The reaction mixture was heated to 60°C for an hour and subsequently worked up in a manner similar to that for dichlorobenzidine, yielding 283 mg of the derivative. The PFP derivative sublimates at 295 to 300°C.

For the standards, a few milligrams of the purified benzidine PFP was weighed and dissolved in dimethylformamide plus ethyl acetate at a 9:1 ratio. Serial dilutions were made with toluene and ethyl acetate at the same ratio. Concentration levels from 129 to 755 $\text{pg } \mu\text{l}^{-1}$ were used. Mass spectra data were obtained on the original and the derivatized benzidine.

Wastewater samples and extraction procedure

Derivatives were prepared from spiked sample extracts in a manner similar to that described above. The water wash procedure described under Micropreparation removes salts and other water-soluble byproducts with very little loss of the derivative; these constituents can cause a high background which will obscure or decrease the responses of the compounds of interest if they are not removed [15, 18].

Approximately 25 mg of sodium thiosulfate was dissolved in 100 ml of the water sample and made basic to pH 10 with 10 M NaOH. The sample was spiked with 2 or 10 μg of a benzidine compound and was extracted four times in a 125-ml separatory funnel with 3.0-ml portions of chloroform. Each 3.0-ml portion of extract was percolated through a 3 cm $\times$ 1 cm column of anhydrous sodium sulfate. After the percolation of the fourth extract, the sodium sulfate layer was washed with 1.0 ml of chloroform. The percolate was evaporated nearly to dryness by a rotary vacuum distillation apparatus at 40°C and 1 mm pressure, and the benzidine compound was converted to the PFP derivative by the micropreparation procedure described above. Percent recoveries from tap water and industrial wastewater are summarized in Tables 1 and 2, respectively.

Calibration

Calibration standards for the compounds of interest were prepared in the following manner. Each isolated derivatized compound was weighed and dissolved in an appropriate solvent, which was toluene-ethyl acetate (9:1) for dichlorobenzidine PFP, and dimethylformamide-ethyl acetate at the same ratio for benzidine PFP, in order to obtain a stock solution concentration of 1 mg ml⁻¹ of the derivatized form. The dimethylformamide-ethyl acetate mixture was necessary to facilitate solution of the benzidine derivative, as toluene-ethyl acetate (9:1) was ineffective at this concentration. Toluene and ethyl acetate at the same ratio were used to prepare serial dilutions, to provide four concentrations with which to construct a calibration curve.

Dichlorobenzidine PFP standards were prepared in concentrations of 170, 340, 510 and 680 pg μl^{-1} as dichlorobenzidine, while benzidine PFP standards were prepared in concentrations of 129, 258, 387 and 580 pg μl^{-1} as benzidine.

TABLE 1

Conversion and recovery yields

<i>N</i> -pentafluoropropionyl (PFP) benzidine derivatives	Pure reagents (Conversion, %)	Tap water runs (Recovery, %)
Benzidine PFP	92 $\pm$ 4 (8) ^a	89.9 $\pm$ 4 (10)
Dichlorobenzidine PFP	94 $\pm$ 4 (7)	92.6 $\pm$ 5 (10)

^aNumbers in parentheses refer to number of test runs.

TABLE 2

Recovery of dichlorobenzidine and benzidine from wastewater

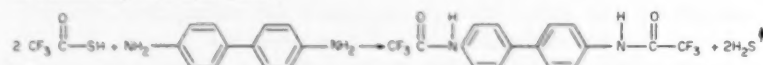
Spike level ^a (μg/100 ml)	Amount recovered (μg/100 ml)	
	Uncorrected ^c	Corrected ^d
<i>Dichlorobenzidine</i>		
0 ^b	0.07 ± 0.03	—
2	1.89 ± 0.21	1.82 ± 0.21
10	9.49 ± 0.77	9.42 ± 0.77
<i>Benzidine</i>		
0 ^b	0.10 ± 0.06	—
2	1.96 ± 0.13	1.86 ± 0.13
10	9.43 ± 1.31	9.33 ± 1.31

^a100 ml of sample spiked. ^bControl was run against a standard containing 19.4 pg μl⁻¹ benzidine PFP and 21.3 pg μl⁻¹ dichlorobenzidine PFP. ^cAverage ± one standard deviation for five samples. ^dCorrection is equal to uncorrected minus average control value for five samples.

RESULTS AND DISCUSSION

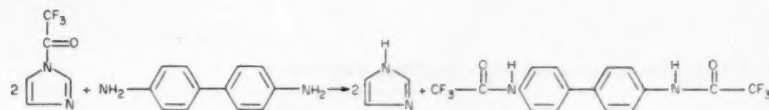
Several derivatization methods were investigated with varying degrees of success. The resulting perfluoropropionamido derivatives of benzidine and 3,3'-dichlorobenzidine exhibited excellent electron capture response and were relatively stable against oxidation and hydrolysis.

Trifluorothioacetic acid was heated with the benzidine dissolved in a toluene-chloroform mixture. At reflux temperature, hydrogen sulfide was evolved with the formation of the trifluoroacetamide of benzidine. The yield ranged from 15 to 90%. This variation was attributed to a difference in reagent quality and to the presence of moisture in the reaction mixture. Moisture will convert the trifluorothioacetic acid to the very strong trifluoroacetic acid, which will readily hydrolyze the product, *N,N'*-bis-trifluoroacetamide, of benzidine.



Derivatization with *N*-methyl-bis-(trifluoroacetamide) was also tried. This reagent quantitatively acylates benzidine but not 3,3'-dichlorobenzidine. The latter compound is not perfluoroacylated, probably because of the bulk hindrance afforded by the chlorine atom in the 3-position adjacent to the amino group of the aromatic moiety. Thus, investigation of this reagent was discontinued.

The third perfluoroacylating reagent used was *N*-trifluoroacetylimidazole. This reagent quantitatively perfluoroacylates the benzidine and dichlorobenzidine. Diphenylhydrazine is about 40% acylated.



Because the *N*-trifluoroacetamide derivatives of benzidine and 3,3'-dichlorobenzidine are hydrolyzable in the presence of moisture, the *N*-pentafluoropropionylamido derivatives (PFP) were prepared with use of the *N*-pentafluoropropionylimidazole (PFPI) reagent. This reagent, being reactive to water, facilitates the removal of water present in solvents. The prepared *N,N'*-bis-(PFP) of benzidine and dichlorobenzidine are far more stable in the presence of moisture than the *N,N'*-bis-trifluoroacetamide of these compounds. The former may be washed with water carefully with less than 5% loss of the derivatives, while under similar water washing techniques, the *N,N'*-bis-(trifluoroacetamides) of these compounds are rapidly hydrolyzed to the original amines in several minutes. Both benzidines react quantitatively with the reagent (PFPI) as shown in Table 1.

Effects of procedural parameters

pH. Samples of wastewater and tap water spiked with benzidine or dichlorobenzidine were extracted at pH 8 or 10 [10]. In acidic solutions, the amino group is protonated and the salt becomes increasingly soluble in water and more difficult to extract. At basic pH, the amino compound is capable of being extracted by a suitable organic solvent. Recovery is higher at pH 10 than at pH 8, as shown earlier [10].

Extraction. Chloroform was a convenient extracting solvent used extensively in this study; its dielectric constant is 4.80 [19]. Methylene chloride was later tested and the results were similar to, or better than, those obtained using chloroform.

Prior to the extraction, the samples were treated for removal of chlorine because chlorine in the water media can chlorinate benzidine in the 3- and 3'-positions via a photochemical reaction, giving 3,3'-dichlorobenzidine. Chlorine can also react with water to yield hypochlorite, which can oxidize the benzidine to yield the diquinonediimine. To minimize these reactions, sodium thiosulfate was added to each tap water and wastewater sample in order to reduce the residual chlorine to the chloride ion. About 25 mg of sodium thiosulfate per 100 ml appears to be sufficient for the reduction of the active chlorine present.

Four extractions were conducted with small portions of chloroform. It should be noted that separatory funnel stoppers were all specially and individually hand ground to promote a tight seal. The extract was concentrated with a rotary vacuum system until the volume was 1.2–2.0 ml.

Reaction volume and reagent. The reaction was conducted with a 10000-fold excess of reagent in a volume of 2–5 ml, in order to promote complete reaction. When the reaction volume was 15 ml or larger, the conversion did not proceed to completion. This was especially true when the prepared

reagent solution aged to six months. Storage in doubly sealed containers at 4°C did not improve stability and this reagent is reactive for only about one month.

Reaction under stress conditions. Because the early literature on the action of acetic anhydride on benzidine shows that the tetra- and tri-substituted forms occur in addition to the expected di-substituted product, the effects of excess of reagent (PFPI), high temperature, and long reaction time were investigated [20]. For a weight ratio of reagent to reactant of 25000:1, at 88°C and with reaction for 5 h, the washed mixture showed that the end product contained no tetra- or tri-substituted product; g.c. showed only one benzidine PFP peak in a chromatogram after 90 min. Moreover, an experiment run with weight ratio of reagent to reactant of 30:1 at 60°C for 1 h yielded essentially 97% of the desired di-substituted product and only 3% of the monosubstituted PFP product, as determined from mass spectra.

Evaporation. The effect of evaporation to dryness under the set vacuum condition of 40°C and 1 mm pressure showed that a water-washed preparation of 10 µg of benzidine as the PFP derivative yields identical response before and after subjecting the derivative sample to the evaporation process. This operation was repeated once and with identical results. It was therefore concluded that there was no loss of the benzidine PFP or dichlorobenzidine PFP derivative when samples were dried under the above vacuum conditions. Only the solvents and most of the reagent are removed in this operation. Salts and PFP derivative remain.

Water washing. Because the salt formed by pentafluoropropionic acid and imidazole as well as some excess of reagent remain with the PFP derivative in the evacuated reaction mixture, conditions for an effective water washing and drying procedure were determined. The first 10 ml of toluene-ethyl acetate (9:1) used to macerate the derivative after evaporation was transferred to a separatory funnel while the next 7.0 ml of toluene-ethyl acetate was used with five drops of water treated initially with sodium thiosulfate to rinse the reaction flask. The flask was then shaken moderately for 4 s, and the contents were transferred to a separatory funnel. After gentle shaking of the contents of the funnel, which were allowed to settle for 10 min, the separated organic layer was passed through the sodium sulphate drying column, followed by 1.0 ml of toluene. An analysis of the contents diluted to volume showed that the response loss of the washed derivative when compared to the unwashed derivative was 4-6%. The base line is flat with a minimal amount of non-interfering solvent front (Figs. 1 and 2). The same results were obtained for dichlorobenzidine and benzidine.

The main peak at 13.5 min and the shoulder at 14.4 min (Fig. 1) were identified by g.c./m.s. as dichlorobenzidine PFP derivatives. Both mass spectra have the same molecular ion and chlorine isotope effects. Their fragmentation patterns are similar, except for a difference in the abundance of certain ions, such as CF_3^+ and $C_2F_5^+$. The two peaks were resolved on a 4% OV-17 column and are interpreted to represent two geometric isomers of dichlorobenzidine PFP (Table 3, see also Fig. 3).

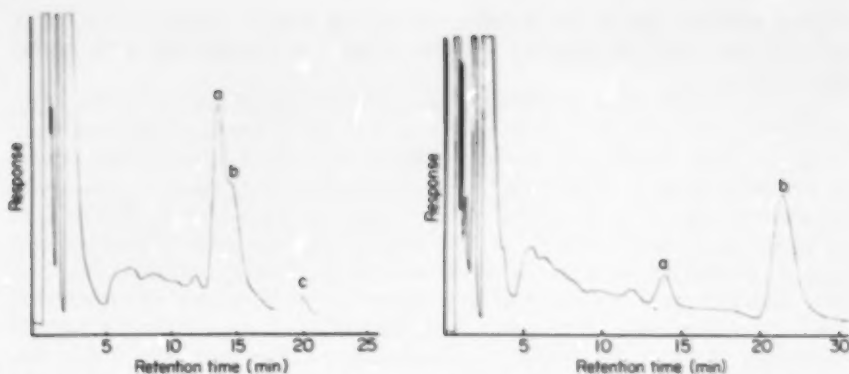


Fig. 1. Chromatogram of pentafluoropropionamidodichlorobenzidine ($t_R = 14.5$ min) recovered from industrial wastewater; 100 pg injected. Peaks a and b are identified as 2 geometric isomers. Conditions: 1- μ l injection volume; carrier gas 5% methane/95% argon; flow rate 20 ml min⁻¹; column 6 ft $\times$ 2 mm i.d. 9:9:2, 3% OV-17 Chromosorb WAW-DMCS 100/120, 3% OV-210 Chromosorb WAW-DMCS 80/100, and 3% OV-225 on Chromosorb WHP 100/120; 210° isothermal; X1024.

Fig. 2. Chromatogram of (b) pentafluoropropionamidobenzidine ($t_R = 22.2$ min) recovered from industrial wastewater; 100 pg injected. Conditions as in Fig. 1. Peak (a) is residual amount of dichlorobenzidine in wastewater as derivative.

TABLE 3

Retention times

(Flow rate 20 ml min⁻¹; 210°C isothermal; 350°C detector temperature; 265°C injector temperature.)

Column ^a	Retention times (min)			
	Benzidine PFP ^c	Dichlorobenzidine PFP ^c	<i>o</i> -Tolidine PFP ^c	Dianisidine PFP ^c
3% OV-17	9.5	10.3	—	—
50/50 3% OV-17/3% OV-210	25.0	23.8	—	—
50/50 3% OV-17/3% OV-225	58.3	20.5	—	—
4% OV-17 ^b	13.8	14.6/26.2 ^d	—	—
40/40/20 3% OV-17/3% OV-210	37.0	17.8	—	—
3% OV-225				
45/45/10 3% OV-17/3% OV-210 ^c	7.1	5.7/6.1 ^d	8.8	11.3
3% OV-225				
45/45/10 3% OV-17	22.2	14.5/16.1 ^d	—	—
3% OV-210				
3% OV-225				

^a3% OV-17—Chromosorb WAW-DMCS 100/120. 3% OV-210—Chromosorb WAW-DMCS 80/100. 3% OV-225—Chromosorb WHP 100/120. ^bMixture: 3% OV-17 on Chromosorb WAW-DMCS 100/120, 5% OV-17 on Chromosorb WAW-DMCS 80/120. ^cRetention time in minutes. ^dTwo isomers separated. ^e225°C isothermal.

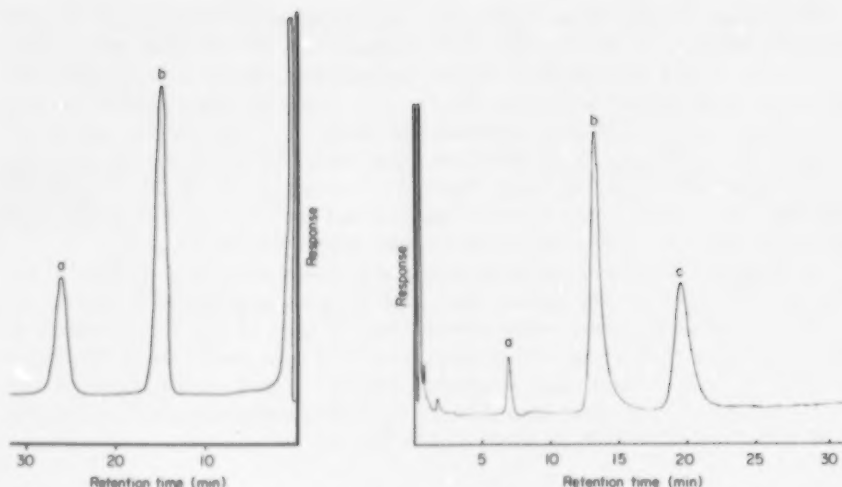


Fig. 3. Separation of two dichlorobenzidine PFP isomers (a and b) on 4% OV-17 column. See Table 3 for conditions.

Fig. 4. Chromatogram of (b) dichlorobenzidine PFP 610 pg ($t_R = 17.8$ min), and (c) benzidine PFP, 445 pg ($t_R = 37.0$ min). (a) is an impurity. Column was 9:9:2 3% OV-17, 3% OV-210, and 3% OV-225. Conditions as in Fig. 1.

Calibration

For four samples of purified dichlorobenzidine PFP between 150 and 700 pg, a least-squares fit of amount found (y) vs. amount added gave a regression equation of $y = 1.036x - 11.0$ and $s_{y,x} = \pm 6.08$ and $r = 0.999$. This equation demonstrates good recovery for this derivative.

Although the dichlorobenzidine PFP derivative was readily prepared, isolated, and recrystallized, the benzidine PFP was not isolated in bulk amount until very late in the study, because it had a tendency to form oils rather than crystals. The calibration curve up to this point was based upon a series of concentrations prepared by the use of the micropreparation method using pure reagents. The least-squares fit for five samples between 200 and 1000 pg gave the equation $y = 0.936x - 80.6$ with $s_{y,x} = \pm 30.5$ and $r = 0.996$. Although this equation demonstrates good linearity, the slope differs from unity and the intercept differs significantly from zero.

The sample compositions were evaluated from calibration data with correction for blank values, which account for the residual amount of the compound or background interference found in wastewater blanks.

Resolution

A 3% OV-17 column did not resolve the benzidine PFP and dichlorobenzidine PFP peaks; however, it showed a linear response over the range of interest and quantification of each individual component was possible.

Two columns, one with 3% OV-210 on Chromosorb WAW-DMCS 80/100 and the other with 3% OV-225 on Chromosorb WHP 100/120, gave retention times longer than 60 min. Shorter retentions were obtained when these materials were mixed with the OV-17. A mixture of equal proportions of 3% OV-17 and 3% OV-210 inverted the order of elution of the two components while keeping both retention times under 30 min; however, the two peaks remained unresolved (see Table 3). A mixture of 3% OV-17 and 3% OV-225 also inverted the order of elution and resolved the two peaks, but the retention time of benzidine was 58 min, which was unacceptable.

A mixture of three-component stationary phases consisting of 3% OV-17, 3% OV-210, and 3% OV-225 in ratio of 2:2:1 gave unacceptably long retention times while the same components mixed in ratio of 9:9:2 gave retention times for both compounds under 30 min and baseline resolution as illustrated in Fig. 4. This column also permitted separation and quantitation of the four benzidine congeners, benzidine PFP, dichlorobenzidine PFP, *o*-tolidine PFP and dianisidine PFP, as illustrated in Fig. 5.

Recovery

The samples used were tap water and diluted industrial wastewater. The wastewater, which was from a dye manufacturing plant, had been acidified to pH 2 to prevent biological and oxidative breakdown of the compounds present. At this pH, the diphenylhydrazine is converted to benzidine. Samples were filtered to remove suspended solids.

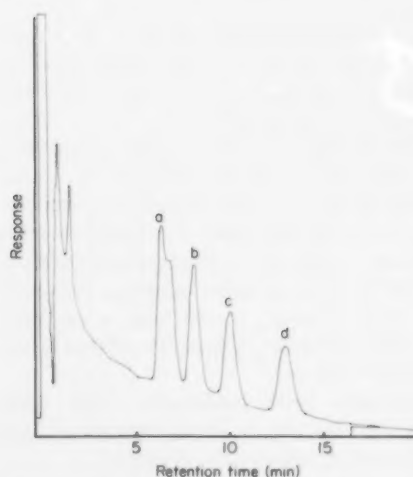


Fig. 5. Chromatogram for four benzidine congeners. Conditions as in Fig. 1 except attenuation X256 and 225°C isothermal. Order of elution: (a) dichlorobenzidine PFP, 80 pg ($t_R = 5.7$ min); (b) benzidine PFP, 74 pg ($t_R = 7.1$ min); (c) *o*-tolidine PFP, 58 pg ($t_R = 8.8$ min), and (d) *o*-dianisidine, 54 pg ($t_R = 11.3$ min).

Three concentration levels were examined, involving 5 to 10 replicates per set. A sample blank was used to determine the contribution of background to the peak height of interest. Samples were spiked with 2 and 10 μg per 100 ml with each compound.

The recovery of dichlorobenzidine was $94.2 \pm 7.8\%$ and $91 \pm 10.7\%$ for the 10 and 2 $\mu\text{g}/100$ ml samples, respectively. The recovery for benzidine was $93.3 \pm 13.1\%$ and $93.0 \pm 6.5\%$ for the 10 and 2 $\mu\text{g}/100$ ml levels, respectively. These values are very similar to those obtained for the recovery of the two benzidines from tap water at the 10 $\mu\text{g}/100$ ml level on ten test runs. The more efficient extraction of dichlorobenzidine relative to benzidine is a result of the lower solubility of dichlorobenzidine in water.

For equal concentrations of benzidine PFP and dichlorobenzidine PFP, the latter gives a higher response due to the two chlorine atoms in the molecule. Minimum detection limits for the dichlorobenzidine and benzidine derivative, were 10 and 50 fg, respectively.

Mass spectrometry

The mass spectral data obtained from pure reagent derivatives and from derivatives of compounds from wastewater extractions were consistent with the assigned structures of the PFP derivatives of benzidine and dichlorobenzidine.

The dichlorobenzidine derivative (m.w. 545) has molecular ions of 544 m/z and 546 m/z because of the isotope effects of the two chlorine atoms and loss of a hydrogen atom. The most abundant ion was 119 m/z which corresponded to a C_2F_3^+ ion. The fragmentation pattern included peaks at 509 m/z and 511 m/z due to loss of a chlorine atom and 397 m/z (loss of a PFP group). Also present at 252 m/z was the dichlorobenzidine molecule minus a hydrogen atom and at 69 m/z corresponding to a CF_3^+ .

The benzidine PFP (m.w. 476) has a molecular ion at 476 m/z . Fragmentation peaks occurred at 329 m/z (loss of PFP) and 182 m/z (loss of two PFP) which is the molecular ion for benzidine. Also present were C_2F_3^+ (119 m/z) and CF_3^+ (69 m/z). The mass spectral data were recorded for both compounds from a wastewater extracted sample and the fragmentation pattern for the benzidine PFP and dichlorobenzidine PFP obtained from the wastewater was similar to those obtained from isolated purified crystals. The mass spectra obtained from wastewater contained more peaks than the purified samples because of background caused by the wastewater matrix.

Conclusion

The derivatization method converts the compound into relatively stable derivatives which permit ease in handling samples and good reproducibility in tests. Reactions were conducted in minimal reaction volumes, using a gross excess of reagent; only disubstitution was observed to occur. Good recovery values can be obtained when employing smaller than usual sample volumes and using four extractions with the extracting solvent.

The four benzidine congeners were resolved easily; in addition, two geometric isomers of dichlorobenzidine are readily separated in the short g.c. column used. The detection limit of the method is below 100 ng l⁻¹ for each benzidine type compound in wastewater. Instrumental detection limit for samples of derivative is about 50 fg.

The authors acknowledge the support and interest given by J. F. Kopp and the review made by J. J. Lichtenberg. The mass spectrometric analysis made by D. Foerst, J. W. Eichelberger, and L. Littlefield is gratefully acknowledged.

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